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Modulation of Colon Tumor Oncogene Expression by Cancer Patient-derived Lipids

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In an effort to understand the role of specific fats on carcinogenesis, we have studied the effects of lipids derived from cancer patients on components associated with the regulation of proliferation. The treatment of tumor cells with patient-derived fats produced increased cell proliferation, as indicated by shorter doubling times. The effects of patient-derived lipids on the expression of *ras, c-jun, c-erbB-2,* and *p53* gene products were examined. The cellular expression of the *ras* proto-oncogene product was increased in both colon tumor cell lines, following lipid treatment. However, c-*jun* proto-oncogene expression was elevated in HT-29 cells and appeared unchanged in SK-Co-1 cells after lipid treatment. Treatment of HT-29 tumor cells with patient-derived fats produced an enhancement of the *p53* gene product, whereas fat treatment reduced *p53* expression in SK-Co-1 tumor cells. Further separation of the patient-derived fats indicated that the amplification of *p53* gene expression in HT-29 cells could be achieved primarily by addition of the diacylglycerides fraction. Addition of the purified fatty acids, comprising the diglyceride fraction, indicated that the fatty acids, 16:1, 18:0, and 18:1, induced the most significant increases in *p53* expression by HT-29 cells. These alterations caused by cancer patient-derived fats are consistent with the loss of normal growth regulation and may explain the epidemiologic association between certain fats and carcinogenesis.

KEY WORDS: **oncogenes, *p53* gene product, lipids, colon cancer**

INTRODUCTION

The majority of human cancers currently are thought to be caused by environmental factors, including nutritional factors, which constitute the most important variable that can be controlled. Over the past 45 years, considerable effort has been made to elucidate the effect of dietary fat on the development of tumors. There are increasing data indicating a direct link between dietary lipids and both the incidence and progression of certain tumors [1–3]. A number of epidemiologic studies have suggested a central role for the consumption of animal fats in the production of these effects [4,5]. Dietary fats, in particular saturated fats, have been implicated in the cause of cancers of the colon, rectum, breast, ovary, and prostate [6,7]. Studies from several laboratories have shown that elevated levels of dietary fat increased the incidence of both spontaneous and chemically induced tumors in experimental animal models [8]. When fat levels are held constant and type of fat is analyzed, there appears to be a link between the degree of saturation of the consumed fast and tumor incidence.

In some cases, epidemiological studies have produced contradicting results, which may be due to methodological problems of dietary assessment, since these studies rarely distinguish between the types of saturated and polyunsaturated fats consumed as part of the diet in humans. Since the characteristics of dietary fats are determined mainly by the types of fatty acids esterified to glycerol, it is likely that the effect of dietary fats on carcinogenesis

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depends on its fatty acid composition. Studies in experimental animals have supported the hypothesis that the fatty acid composition of dietary fat is the primary determining factor in carcinogenesis. [9].

Investigations have evaluated the role of certain fatty acids in the regulation of protein synthetic events [10,11]. These studies indicated that certain fatty acids are capable of altering the activity and relocation of protein kinase C, which can initiate uncontrolled cell proliferation. In fibroblast cell lines, transfected with an activated human c-H-ras oncogene and subsequently cultured in medium supplemented with fatty acids (20–80 μM), a marked enhancement in the number of transformed foci was obtained, when the cultures were incubated with myristic, palmitic, or stearic acids [12]. The fatty acid levels and types also have been reported to modify the membrane phospholipid composition, 1,2-sn-diacylglycerol (DAG) levels, and protein kinase C activity [13]. Increased level of DAGs and enhanced activity of protein kinase C have been demonstrated to play important roles in tumor promotion. Thus the elevation in these parameters produced by high fat diets may be critical in the higher cancer rates observed with these diets.

Whereas experimental and epidemiological studies provide evidence that a high fat diet or diets rich in certain fatty acids enhance many tumor types by acting at the tumor promotion stage, the mechanism(s) for this enhancement are not known. However, based on these epidemiological studies indicating an enhanced "malignancy" of tumors in individuals consuming specific fats, this study examines the effects of patient-derived fats from individuals with cancer on the expression of components associated with progressive disease (the proto-oncogenes, *ras, jun, c-erbB-2,* and the tumor suppressor gene product, *p53*).

MATERIALS AND METHODS

Isolation of Lipid Fraction From Biological Fluids of Gynecologic Cancer Patients

To study the effects of patient-derived lipids, lipids were obtained either from ascitic fluid specimens from individuals with advanced ovarian cancer or from normal peritoneal fluid of a nontumor-bearing female patient undergoing abdominal surgery. The fluids were clarified by centrifugation at 100,000xg for 1 hour. Lipids were extracted by the modified procedure of Bligh and Dyer [14]. The lipid suspension from each patient (or volunteer) was brought to a volume of 1 ml with 0.1 M formic acid with 1% NaCl, and lipids were isolated by performing a two-step extraction with chloroform:methanol:water. Initially, lipids were extracted with chloroform:methanol:water (1:2:0.8), and then chloroform:water were added to form a two phase system (1:1:0.9). The lipids contained in the chloroform phase were concentrated in a rotary evaporator.

This unfractionated lipid population was utilized for studies of cell proliferation and initially on proto-oncogene expression. Subsequently, 500 μg of extracted lipid from a patient with stage III ovarian serous papillary adenocarcinoma (Patient 3) was separated by thin layer chromatography (TLC) using a Silica gel G plate. The lipids were eluted with chloroform:methanol:water (1:1:0.2, v/v) and following separation, the lipid spots were visualized with iodine vapor. The spots corresponding to phospholipids, free fatty acids, and mono-, di-, and triglycerides (based on simultaneously run standards) were scraped from the plate and the groups were eluted and dried. The total fatty acid concentration of this sample was determined by gas chromatographic analysis following saponification. Each sample was separated on a DuraBond 225 column using a Hewlett-Packard 5890 gas chromatograph. The fatty acids were separated using a standard temperature ramp of 165° to 226° at 3°/minute.

Culturing of Tumor Cells and Supplementing With Patient-derived Fats

The alterations in in vitro tumor growth and the expression of specific cellular proteins, induced by patient-derived fats, were investigated using two cultured human colon tumor cell lines, HT-29 and SK-Co-1. The colon tumor cell lines were obtained from the American Type Culture Collection (Rockville, MD). These tumor cells were grown in Dulbecco's modified Eagle's medium, supplemented with 2% delipidated fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 200 mM L-glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin in a humidified 5% CO_2 atmosphere. To study the effects of patient-derived lipids, the lipid layer of the ascitic fluid specimen from Patient 3 (Table I) was obtained as described above and the total fatty acid concentration added to the culture medium was 50 μg/ml. In previous studies [12], the in vitro consequences of lipids on oncogene expression were examined with individual fatty acids at 20–80 μM. The cell cultures were supplemented with the fatty acid mixture for two passages (7 days) prior to studies. Cells were additionally cultured with individual purified fatty acids (Sigma Chemical Co., St. Louis, MO), previously identified as present in the diglyceride fraction of the ascites. Each fatty acids was added (see Fig. 3) at a concentration proportional to its concentration in the original ascitic fluid [15].

Determination of Cell Growth Rates

Control (untreated) cells and fat treated cells were removed from culture flasks by treatment with trypsin/EDTA. Cells (2×10^4) were plated on 35 mm^2 tissue culture dishes in complete media supplemented with patient-derived fats. After 24, 48, and 72 hours, triplicate dishes for each condition were harvested by treatment

TABLE I. Distribution (Percent) of Patient-derived Fat Into Phospholipids, Free Fatty Acids, Monoglycerides, Diglycerides, and Triglycerides*

	Percent of total fluid-derived lipids			
	Control	Patient 1	Patient 2	Patient 3
Phospholipids	17.0	4.1	18.8	15.6
Free fatty acids	6.3	36.4	34.0	28.8
Monoglycerides	0.3	1.3	1.9	1.8
Diglycerides	1.3	5.3	3.9	2.9
Triglycerides	75.1	52.9	41.4	51.4
Total fats analyzed (μg)	489.0	505.4	524.3	483.1

*Approximately 500 μg of fluid-derived fat was obtained from either a normal volunteer or from the ascites of three patients with advanced ovarian cancer. This fat was extracted as described in Materials and Methods and separated by thin layer chromatography. Each group was removed by scraping the appropriate spot on the plate and the amount of these group was quantitated following re-extraction.

with trypsin/EDTA (ethylenediamine tetra-acetic acid), and the total number of cells were determined. The mean cell number was plotted versus time (hours), and the time required for the cells to double was detemined.

Western Blot Analysis for Tumor-Associated Alterations

Control and fat treated cell cultures were grown and in exponential phase of growth were harvesed and processed for Western blot as described by Brown et al. [16]. Cells were lysed in 1% NP-40, 500 mM NaCl, 50 mM Tris (pH 7.5), 1 mM DTT (dithiothreiol), and 1 mM PMSF (phenylmethylsulfonyl fluoride). Protein concentrations were determined by the Bio-Rad assay and were adjusted to identical concentrations. Solubilized proteins were separated by SDS-polyacrylamide gel electrophoresis on either a 7.5% (for c-*erbB*-2) or 12% (for *ras, p53,* and c-*jun*) acrylamide gel and transferred onto nitrocellulose membranes (Biotrace NT, Gelman Sciences, Ann Arbor, MI) by semidry electroblotting in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol). The blots were blocked with 5% nonfat dry milk, 50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, and 1 mM DTT for 4 hours at 4°C, and probed overnight at 4°C in the same buffer with 1 mg/ml of the specific monoclonal antibody. The specific antibodies used in this study were anti-*p53, -ras,* c-*erbB2,* and *jun* (Oncogene Science). After this incubation, the membranes were washed three times in 0.1% Tween-20 in PBS (2-mM sodium phosphate-buffered saline) for 15 minutes at room temperature. The blots were then incubated in blocking buffer with anti-mouse IgG conjugated with horseradish-peroxidase for 45 minutes at room temperature. These immunoblots were washed again three times in 0.1% Tween-20 in PBS, after which bound complexes were visualized by enhanced chemiluminescence (ECL, Amersham Life Sciences, Arlington Heights, Il.). The resulting bands were compared to prestained molecular weight standards run simultaneously to verify the appropriate molecular weight.

Statistical Analysis of Data

Determination of cell proliferation was performed in quadruplicate and differences were analyzed by two-tailed Student's t-test, using GraphPad Instat software. Studies showing the effects of lipids on proto-oncogene expressions were performed at least twice and representative results are presented. The levels of fat subgroups were determined in duplicate samples, and figures presenting the fat levels and distribution are the average of these results.

RESULTS

Distribution of Major Lipid Groups in Patient-derived Fatty Acids

To investigate the effects of patient-derived lipids on certain parameters of tumor cells, lipids were isolated from patients with advanced cancer who were undergoing treatment at the James Graham Brown Cancer Center. The distribution and concentrations of fats were determined for these samples following extraction, saponification, and separation by thin layer chromatography (TLC). Lipids were isolated from these patients with advanced cancer and the distributions of fats were determined for 500 μg aliquots of these samples following separation by TLC (Table I). There appeared to be four consistent differences between patient-derived fats and normal volunteer-derived fats: both mono- and diglycerides as well as free fatty acids were elevated, whereas triglycerides were reduced.

Effect of Patient-derived Fats on Cell Proliferation

To evaluate the effect of fats on cell proliferation, the doubling rates of the two colon tumor cell lines were determined following growth in the presence and absence of 50 μg/ml lipid from Patient 3 (Table II). The doubling times for HT-29 and SK-Co-1 cells were 16.8 and 24.6 hours, respectively. After treatment with patient derived fats for two passages, the doubling times for HT-29 and

TABLE II. Cell Doubling Times of Human Colon Tumor Cell Lines, HT-29 and SK-Co-1, in Presence and Absence of Patient-derived Fats*

	Untreated	Fat-treated	*P* value
HT-29	16.8 ± 0.7	14.4 ± 0.5	0.0009
SKC0-1	24.6 ± 0.8	22.8 ± 0.6	0.0158

* Cell cultures supplemented the patient's fats, 50 μg/ml, were cultured for two passages prior to studies. Quadruplicate wells were harvested at each time point for determination of doubling times.

SK-Co-1 cells were decreased to 14.4 (14%) and 22.8 (7% reduction) hours, respectively.

Effect of Patient-derived Fats on Expression of *ras*, *jun*, and c-*erbB2* Oncogene Products

After adjusting the samples to identical protein concentrations, alterations in the expression of *ras*, *jun*, and c-*erbB2* proto-oncogene products were analyzed by Western immunoblotting. The cellular expression of the *ras* oncogene product was enhanced in both colon tumor cell lines, following fat treatment (Fig. 1A). The cellular expression of the *c-jun* oncogene product was also enhanced by fat treatment in the HT-29 tumor cell line, following fat treatment (Fig. 1B). The c-*erbB2* oncogene product was not detectable in HT-29 and fat treatment did not alter its expression, whereas in SK-Co-1 cells, c-*erbB2* was expressed and fat treatment reduced its expression (Fig. 1C).

Effect of Patient-derived Fats on Expression of the Tumor Suppressor Protein, *p53*

Alterations in *p53* antigen expression was analyzed by western immunoblotting. Treatment of the ovarian tumor cell lines with fats produced variable effects (Fig. 2). In the HT-29 cells, treatment with fats induced the anti-p53 reactive bands. Subsequent immunoprecipitation studies in these HT-29 cells indicated that this induced p53 antigen represented the mutated forms of the protein (data not shown). SK-Co-1 cells expressed p53 band in the untreated controls and treatment with lipids reduced its expression. To determine the group of lipids responsible for the induction of *p53* in HT-29 cells, the fats were separated into free fatty acids, phospholipids, monoglycerides, diglycerides, and triglycerides. Treatment of HT-29 cells with the unfractionated fats and the resulting fractions revealed that the primary inducing activity appears to be associated with diglycerides, with lesser levels associated with mono-and triglycerides (Fig. 3).

Assessment of Individual Fatty Acids on Alterations in *p53* Tumor Suppressor Gene Expression

To determine the specific fatty acids responsible for the observed alterations in *p53* expression, the purified

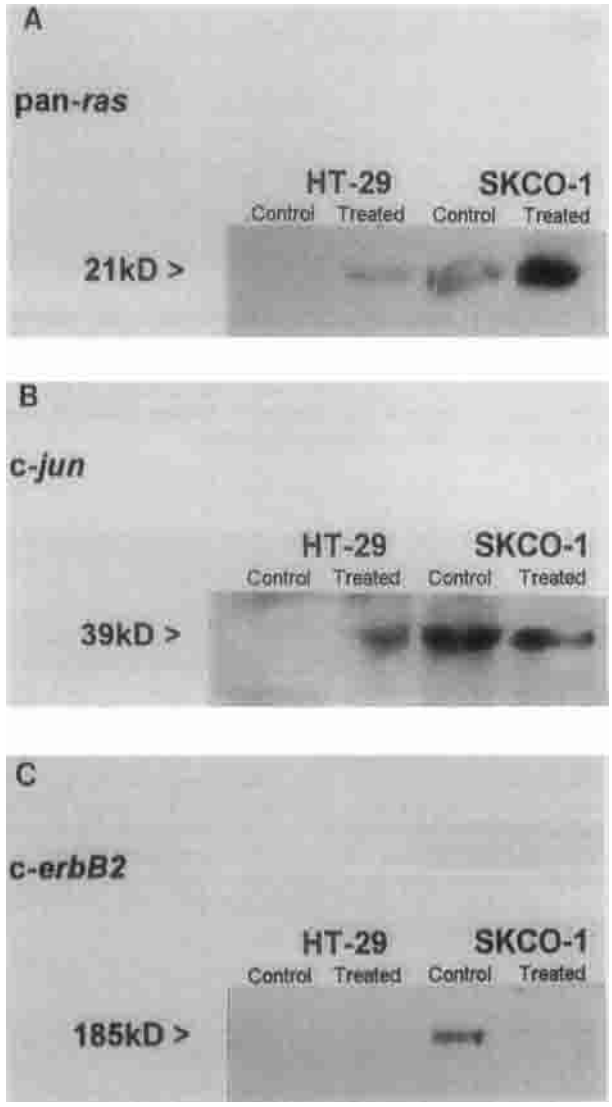

Fig. 1. Effect of in vivo mobilized fats on the expression of the proto-oncogenes: *ras* **(A)**, c-*jun* **(B)**, and c-erbB2 **(C)** gene products by human colon tumor cell lines, HT-29 and SK-Co-1. Cell cultures supplemented the patient's fatty acids, 50 μg/ml, were cultured for two passages prior to studies. Alterations in *ras*, c-*jun*, and c-*erbB-2* antigen expressions were analyzed by Western immunoblotting.

fatty acids (identified in the diglyceride fraction of the patient-derived ascitic fluid) were incubated with HT-29 cells for 7 days and changes in *p53* expression were analyzed by Western immunoblot (Fig. 4). These individual fatty acids were added at a concentration proportional to their concentration in the unfractionated fluid. Addition of five fatty acids (16 : 1, 18 : 0, 18 : 1, 20 : 3, and 20 : 4) appeared to significantly enhance *p53* protein levels; however, the fatty acids, 18 : 0 and 18 : 1, produced the greatest enhancement.

DISCUSSION

Recognizing that lipid metabolism is dramatically altered in many cancer patients, particularly in terms of

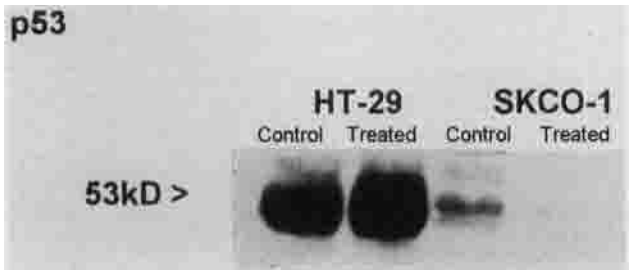

Fig. 2. Effect of unfractionated in vivo derived fats on the expression of *p53* gene product by human colon tumor cell lines, HT-29 and Sk-Co-1. Cell cultures, supplemented with patient-derived lipids (50 μg/ml), were cultured for two passages (7 days) prior to studies. Alterations in p53 antigen expression were analyzed by Western immunoblotting at a standardized cellular protein concentration.

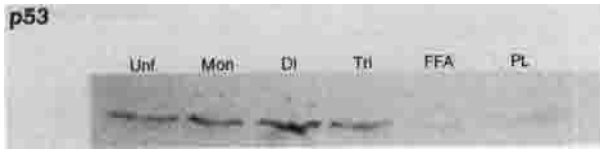

Fig. 3. Effect of phospholipids, free fatty acids, monoglycerides, diglycerides, and triglycerides obtained by fractionation of in vivo fats (patient-derived) on the expression of *p53* gene product by the human colon tumor cell line, HT-29. Cell cultures, supplemented with the patient-derived lipid fractions, were cultured for two passages prior to studies. Alterations in p53 antigen expression were analyzed by western immunoblotting. Fractions added were Unf = unfractionated (50 μg/ml), Mon = monoglycerides (1 μg/ml), Di = diglycerides (2 μg/ml), Tri = triglycerides (20 μg/ml), FFA = free fatty acids (17 μg/ml), PL = phospholipids (10 μg/ml).

Fig. 4. Effect of purified fatty acids on the expression of *p53* gene products by the human colon tumor cell line, HT-29. Cell cultures supplemented the purified fatty acids, identified as being present in the diglyceride fraction, where cultured for two passages prior to studies. Alterations in p53 antigen expression were analyzed by Western immunoblotting. The fatty acids were added at a concentration proportional to that in the original ascites: 14 : 0 at 1 μg/ml, 16 : 0 at 10.8 μg/ml, 16 : 1 at 1 μg/ml, 18 : 0 at 2.5 μg/ml, 18 : 1 at 18.5 μg/ml, 18 : 2 at 12.4 μg/ml, 18 : 3 at 1.3 μg/ml, 20 : 0 at 0.25 μg/ml, 20 : 1 at 0.5 μg/ml, 20 : 3 at 0.6 μg/ml, 20 : 4 at 1 μg/ml, and 22 : 6 at 0.5 μg/ml.

enhanced utilization, and that these effects appear to be the direct consequence of the tumor or tumor produces, we have studied lipid metabolism associated with tumors. We have previously demonstrated that certain tumors express and release a lipolysis promoting factor (LPF) [17] and this factor can mobilize stored fatty acids from normal adipocytes, both in vitro and in vivo. These stored fats are continuously turning over and exchanging their component fatty acids with those in the circulating lipid compartment. Further, in vitro studies have indicated the tumor represents a "sink" for elevated circulating lipids, resulting from the action of LPF [18,19]. Therefore, the role of in vivo patient-derived fats in altering the characteristics of tumor cells was studied.

Since lipid substrates appear to represent a preferred energy source for tumors, it was postulated that these in vivo derived fats might enhance doubling times. The doubling times were 16.8 and 24.6 hours for HT-29 and SK-Co-1, respectively, in controls, whereas the cultures treated with patient-derived fats have doubling times of 14.4 (HT-29) and 22.8 (Sk-Co-1) hours (Table II). Cell proliferation is regulated by controlled expression of proto-oncogenes. As part of this study, the effect of patient-derived fats were defined on three broad classes of proto-oncogenes: class II (hormone receptor-like), c-*erbB-2;* class III (intercellular transducers), c-*ras;* and class IV (nuclear transcription regulators), c-*jun* and *p53*.

When analyses of the proto-oncogenes were performed, the difference in the number of cells was normalized to account for the observed increase in cell proliferation. One consistent finding was that the cellular expression of the *ras* oncogene product was enhanced in both SK-Co-1 and HT-29 cells, following lipid treatment (Fig. 1A). In contrast, the c-*jun* oncogene product, which can be modulated by *ras,* was enhanced in HT-29 cells and decreased in SK-Co-1 cells (Fig. 1B). c-*erbB*-2 was not expressed by HT-29 cells and following lipid treatment, no induction was observed (Fig. 1C). Sk-Co-1 cells expressed c-*erbB-2*; however, lipid treatment caused the loss of this expression. Thus fat treatment did not produce a generalized amplification of cellular proteins in either of these cell lines, except in the induction of the *ras* oncogene product.

Treatment of SK-Co-1 cells with fats decreased p53 expression; however, treatment of HT-29 cells with patient-derived fats produced a dramatic amplification of the *p53* gene product (Fig. 2). Immunoprecipitation studies have demonstrated that this enhanced *p53* expression in HT-29 represented the mutated form. To determine the lipid fractions responsible for this induction, lipid fractions were separated by high performance thin layer chromatography into mono-, di-, triglycerides, free fatty acids, and phospholipids. Each of these fractions was added at a concentration equal to that in the original sample. The study demonstrated that diglycerides, followed by monoglycerides and triglycerides, contained the material that caused the greatest alteration in *p53* expression (Fig. 3). Previous studies defined the composition of the diglyceride fraction. Addition of purified fatty acids present in the diglyceride fractions demonstrated that only certain fatty acids amplified the expression of *p53*. The fatty acids, 16 : 1, 18 : 0, and 18 : 1. produced

the greatest induction of *p53* expression by HT-29 cells (Fig. 4).

A number of epidemiologic studies have demonstrated the link between certain dietary lipids and the development and progression of some tumors, including breast, ovarian, and colon cancers. This study attempts to address the underlying mechanisms of this association. Since the in vivo mobilized lipids, which are present in the serum and ascites of cancer patients, reflect the long-term dietary lipid intake, the consequences of patient-derived lipids on tumor growth and expression of certain oncogenic proteins were evaluated. The use of ascites derived from gynecologic cancer patients was simply as a source of lipids from individuals with advanced cancer. The epidemiologic link between lipids and cancer is strengthened by the use of purified fatty acids. These findings suggest that the presence of certain fatty acids can enhance the expression of oncogenic products necessary for tumor progression.

CONCLUSION

Fat-treatment of HT-29 and Sk-Co-1 cells exhibit different, in some cases opposite, consequences. The rationale for this differential effect is unclear, but could result from aberrant expression of fatty acid binding proteins, expression of mutated versus wild-type gene products (in the case of *p53*), or differences in the regulatory pathway modulated. The alteration in *ras* expression appears to be a common, fat-regulated pathway modulated. The alteration in *ras* expression appears to be a common, fat-regulated site. Since activation of *ras* products can influence multiple regulatory pathways, such as *jun/fos* and NF_KB, the modified pathway in HT-29 cells may be distinct from that in SK-Co-1 cells. Components of the NF_KB pathway have not been addressed, as of yet. To address these potential explanations, differential display is currently being used to identify all alterations induced by these fats.

Numerous models of carcinogenesis have demonstrated that c-*ras* proto-oncogenes are common targets for the initial events of carcinogenesis. Based on the two-step model of transformation, there must be a link between the activation of class III oncogenes and subsequent induction of class IV oncogenes. Our data suggest this association in both amplification of class III (c-*ras*) and class IV proto-oncogenes (c-*jun* and *p53*). Thus it would appear that certain fats can play an important role in carcinogenesis. Current investigations are evaluating whether this "carcinogenic" effect is important only in the progression of existing tumors, as seen here, or if similar effects are produced in normal cells and may represent a first (initiation) step or second (promotion) transforming step. The data presented here provide information on the role of lipids, with specific fatty acids identified, their metabolism, and consequence in carcinogenesis with respect to oncogene expression. These aberrations induced by the treatment of certain tumor cells with in vivo derived fats are consistent with the loss of growth regulation and may provide an explanation for the epidemiological data correlating fats and the progression of tumors.

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